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The DNA Binding Site of the Dof Protein NtBBF1 Is Essential for Tissue-Specific and Auxin-Regulated Expression of the *rolB* Oncogene in Plants

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The Dof proteins are a large family of plant transcription factors that share a single highly conserved zinc finger. The tobacco Dof protein NtBBF1 was identified by its ability to bind to regulatory domain B in the promoter of the *rolB* oncogene. In this study, we show that the ACTTTA target sequence of NtBBF1 in domain B is necessary for tissue-specific expression of *rolB*. β-Glucuronidase (GUS) activity of tobacco plants containing a *rolB* promoter–*GUS* fusion with a mutated NtBBF1 target sequence within domain B is almost completely suppressed in apical meristems and is severely abated in the vascular system. The ACTTTA motif is shown here also to be one of the *cis*-regulatory elements involved in auxin induction of *rolB*. The pattern of *NtBBF1* expression in plants is remarkably similar to that of *rolB*, except in mesophyll cells of mature leaves, in which only *NtBBF1* expression could be detected. Ectopic expression of *rolB* in mesophyll cells was achieved by particle gun delivery if the NtBBF1 binding sequence was intact. These data provide evidence that in the plant, a Dof protein DNA binding sequence acts as a transcriptional regulatory motif, and they point to NtBBF1 as the protein involved in mediating tissue-specific and auxin-inducible expression of *rolB*.

INTRODUCTION

Infection of dicotyledonous plants with *Agrobacterium rhizogenes* results in hairy root disease (Elliot, 1951). Hairy root causes an abundant proliferation of neoplastic roots containing T-DNA from the root-inducing (Ri) bacterial plasmid (Chilton et al., 1982; Spanò et al., 1982; White et al., 1982; Willmitzer et al., 1982). In the T-DNA sequence, 18 open reading frames have been identified (Slightom et al., 1986). Four of these, termed the *rol* oncogenes, coincide with genetic loci previously localized by transposon mutagenesis (White et al., 1985). Among the *rol* oncogenes, *rolB* is the only one that, when inactivated, totally suppresses root induction by *A. rhizogenes* (White et al., 1985). In addition, *rolB* is the only individual gene capable of inducing growth of transformed roots for all plants tested (Cardarelli et al., 1987; Spena et al., 1987; Capone et al., 1989). More recently, in cultured thin cell layers (Tran Thanh Van et al., 1974), the stimulation of meristem formation has been shown to be the primary effect of *rolB* (Altamura et al., 1994). Expression of *rolB* greatly increases the sensitivity of transformed plant cells to auxin (Spanò et al., 1988; Maurel et al., 1991), and tyrosine phosphatase activity has been associated with the RolB protein when it is expressed in *Escherichia coli* (Filippini et al., 1996), suggesting that RolB may act by perturbing the auxin signal transduction pathway.

The expression of *rolB* in transgenic plants also has been extensively analyzed and found to be strongest in root and shoot meristems (Schmülling et al., 1989; Maurel et al., 1990; Altamura et al., 1991; Capone et al., 1991), which is in accord with an effect of the oncogene on meristem induction. Expression was also detectable in the vascular system. Moreover, *rolB* has been found to be induced by auxin (Maurel et al., 1990, 1994; Capone et al., 1991, 1994). A number of *cis*-regulatory domains have been identified in the promoter of *rolB.* Different combinations of these domains direct expression of the gene in different populations of cells in the (root) meristem (Capone et al., 1994). In particular, domain B, which is localized between positions −341 and −306, is essential for expression of *rolB* in all cells of the apical meristems and to enhance expression in the vascular system. This domain also is necessary for the gene's auxin responsiveness (Capone et al., 1991, 1994).

The gene encoding a nuclear protein specifically binding to domain B was subsequently isolated from a tobacco expression library (De Paolis et al., 1996). The protein NtBBF1 is characterized by a single C_2-X_{21}-C_2 zinc finger domain of a novel type, with this domain specifically binding to the ACTTTA motif within domain B (De Paolis et al., 1996). Most interestingly, this new single zinc finger domain has been identified in a number of other tobacco proteins (De Paolis

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et al., 1996) and in proteins encoded by genes of such distantly related plants as Arabidopsis (Zhang et al., 1995; De Paolis et al., 1996), maize (Yanagisawa and Izui, 1993; Vicente-Carbajosa et al., 1997), pumpkin (Kisu et al., 1995), and barley (Mena et al., 1998). In all of these proteins, a 52–amino acid segment designated the Dof domain (Yanagisawa, 1995), which encompasses the 29–amino acid zinc finger region, is strikingly conserved.

The wide distribution of Dof proteins within (and only within) the plant kingdom and the unusual conservation of the Dof domain coupled with the wide divergence of sequences outside the 52–amino acid domain suggest a crucial role for these proteins in regulating different functions typical of and universally relevant to plants. By means of transient expression assays with protoplasts, it has been shown recently that the maize Dof1 and Dof2 proteins are indeed transcription factors. It also has been suggested that they may mediate light gene regulation in leaves (Yanagisawa and Sheen, 1998). Another member of the maize Dof gene family is PBF (for prolamine box binding factor). This protein binds to the prolamine box in zein gene promoters (Vicente-Carbajosa et al., 1997). In Arabidopsis, the Dof protein OBP1 (for octopine synthase [OCS] binding factor [OBF] binding protein) binds to the promoter of a glutathione *S*-transferase gene (Zhang et al., 1995; Chen et al., 1996). In pumpkin, the Dof protein AOBP binds to an ascorbate oxidase gene (Kisu et al., 1995). Recently, transient expression experiments in developing barley endosperm showed that the BPBF Dof protein is capable of activating the B-hordein promoter (Mena et al., 1998). Thus far, however, little is known about the biological role in plants of any of the members of this new and widely conserved family of regulatory proteins.

In this study, we investigated the role of the tobacco Dof protein NtBBF1 in the regulation of the oncogene *rolB* in plants. We show that the DNA binding sequence of NtBBF1 in the *rolB* promoter is essential for tissue-specific expression as well as for conferring auxin inducibility to the oncogene. We also show that the gene encoding NtBBF1 is expressed in a pattern similar to that of *rolB*, that is, most strongly in the apical meristems. In the only tissue in which *NtBBF1* is expressed and *rolB* is not, namely, in the mesophyll, ectopic expression of the latter by means of particle gun delivery depended on the presence of the NtBBF1 target sequence. These data provide strong evidence that NtBBF1 plays a pivotal role in regulating the expression of *rolB*, and they will help in investigations of the mechanism of auxin induction and in the identification of plant genes possibly involved in meristem formation and morphogenesis.

RESULTS

Band-shift experiments with a set of synthetic oligonucleotides representing different mutated versions of regulatory domain B (spanning nucleotides −341 to −306 from the translation start codon) of the *rolB* promoter allowed us to identify the sequence ACTTTA as the in vitro binding site for the tobacco Dof protein NtBBF1 (De Paolis et al., 1996). To investigate the function of this *cis* element in the B341 promoter (Capone et al., 1991, 1994), a mutated version of the promoter, in which the ACTTTA motif (nucleotides −312 to −307) was changed to TGATTA, was fused to the β-glucuronidase (*GUS*) reporter gene (Jefferson et al., 1987). Figure 1 shows the mutated construct B341M3–*GUS* and the B341–*GUS* and B306–*GUS* constructs (Capone et al., 1991, 1994) used in this work.

Target Sequence of NtBBF1 Is Essential for Expression of *rolB* in Apical Meristems

To assess the role of the ACTTTA target sequence of NtBBF1 in mediating control of *rolB* expression in plants, we transformed tobacco with the unmodified B341–*GUS* (Capone et al., 1991, 1994) and the mutated B341M3–*GUS* constructs. We had shown previously that deletion of all of domain B results in suppression of *rolB* expression in apical meristems and a substantial reduction in the vascular system (Capone et al., 1991). We first compared the apical meristems of B341–*GUS* and B341M3–*GUS* plants, as shown in Figures 2A, 2D, and 2G and Figures 2B, 2E, and 2H, respectively, in which results obtained with plantlets 3 weeks after germination are reported. In Figure 2A, the typical pattern of his-

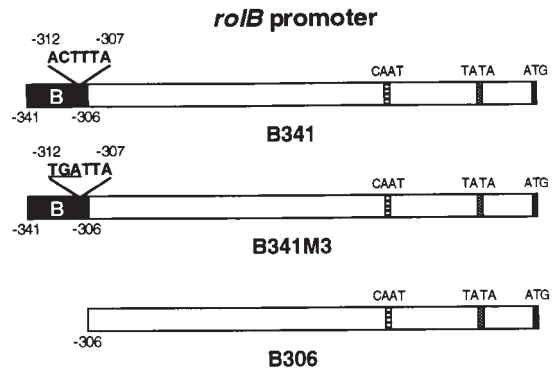

Figure 1. *rolB* Promoter–*GUS* Gene Fusions.

The bars represent the versions of the *rolB* promoter used for transcriptional fusions with the *GUS* reporter gene. These fusions in plasmids pBI and pUC18 were used for plant transformation and particle gun delivery assays, respectively. The numbers indicate nucleotide positions from the *rolB* translation start codon. Regulatory domain B (B) is represented by the black boxes. The binding sequence of the protein NtBBF1 and its mutated version are indicated as inserts. The positions of the CAAT and TATA boxes of the *rolB* promoter are also shown.

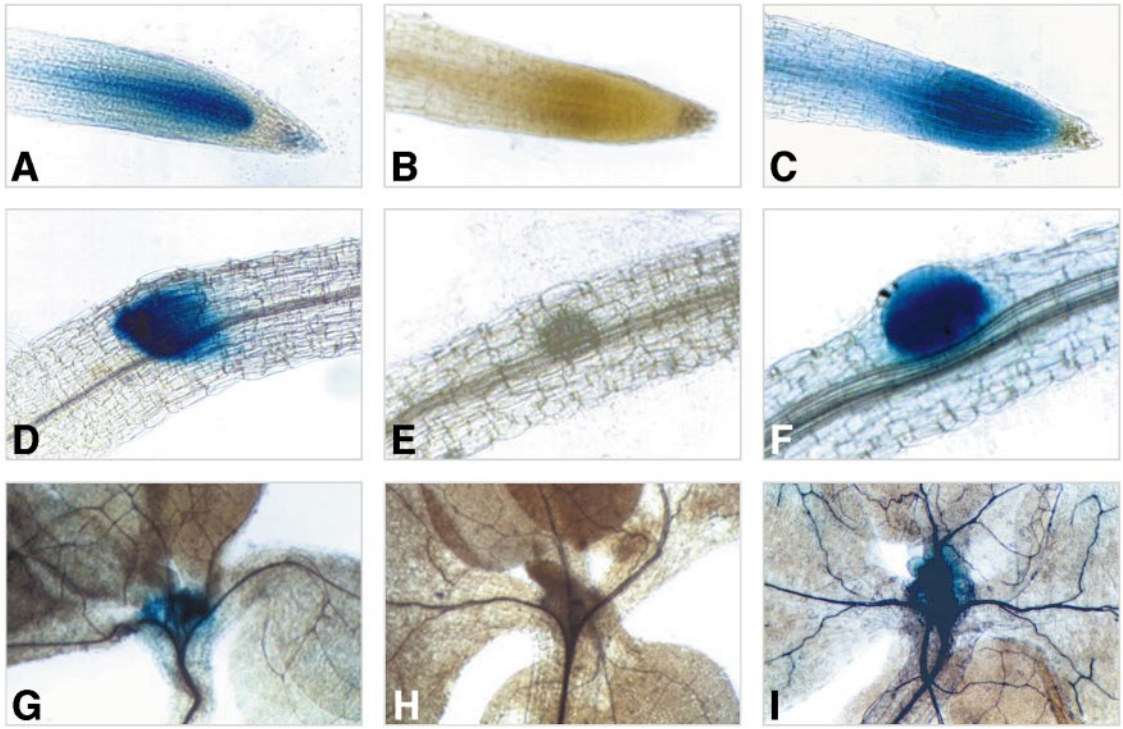

Figure 2. Mutation of the Binding Sequence for NtBBF1 in B341M3–*GUS* Suppresses *rolB*-Driven B341–*GUS* Expression in Apical Meristems.

The *NtBBF1* gene (BBF814–*GUS*) is most strongly expressed in apical meristems of tobacco plants. Sections from T_2 plantlets 3 weeks after germination are shown.

(A), **(D)**, and **(G)** Activity of the B341–*GUS* construct in the primary root apex, secondary root primordium, and shoot apex, respectively.

(B), **(E)**, and **(H)** Activity of the B341M3–*GUS* construct in the primary root apex, secondary root primordium, and shoot apex, respectively.

(C), **(F)**, and **(I)** Activity of the BBF814–*GUS* construct in the primary root apex, secondary root primordium, and shoot apex, respectively.

tochemical staining of the primary root apex of a plant due to the activity of the B341–*GUS* construct is shown. As previously reported (Capone et al., 1991, 1994), this construct is very active in the dermatogen and in meristematic cells of the cortex and of the vascular cylinder. In sharp contrast, Figure 2B shows that the primary root apex of a transgenic tobacco plant of the same age containing the construct B341M3–*GUS* is totally unstained in most roots and only occasionally very faintly stained, indicating that the activity of the *rolB* promoter in root meristems is almost completely suppressed by the modification of the target sequence of NtBBF1. The same holds true for the primordia of lateral roots in which strong GUS staining is shown by B341–*GUS* plants, whereas B341M3–*GUS* plants show no reporter gene activity, as illustrated by Figures 2D and 2E, respectively.

The target sequence of NtBBF1 also is essential for gene expression in the shoot apical meristem. Whereas the unmodified B341 promoter drives the strong GUS expression shown in Figure 2G, B341M3–*GUS* plants, illustrated in Figure 2H, do not show any appreciable GUS-specific staining. The same differences in GUS activity between B341–*GUS* and B341M3–*GUS* plants in root and shoot apical meristems were observed in more mature plants grown in vitro and in the greenhouse (data not shown).

Mutation of the NtBBF1 Target Sequence Also Affects *rolB* Expression in the Vascular System

Although its expression is strongest in apical meristems, *rolB* also is active in the phloem and in the phloem and xylem parenchyma (Altamura et al., 1991; Capone et al., 1991). However, in young plants in particular, the level of GUS activity driven by deletions of the *rolB* promoter shorter than 341 bp is rather low in the vascular system and not easily detectable by histochemical staining (Capone et al., 1991, 1994). Therefore, we compared the activity of the B341–*GUS*, B341M3–*GUS*, and B306–*GUS* constructs by using fluorometric assays with total extracts from different organs.

Figure 3 shows that the level of fluorescence due to both the deleted B306–*GUS* and the mutagenized B341M3–*GUS* constructs drops dramatically (but is still measurable) in all organs of transgenic plants, as compared with B341–*GUS*.

Target Sequence of NtBBF1 Is Involved in Auxin Responsiveness of the *rolB* Promoter

It had been shown previously that the promoter of *rolB* is activated by auxin (Maurel et al., 1990, 1994; Capone et al., 1991, 1994) and that domain B is necessary for this hormonal response (Capone et al., 1991, 1994). To assess the relevance for auxin induction of the ACTTTA sequence contained in domain B, we incubated small leaf explants devoid of primary and secondary veins (miniexplants) from in vitro–grown B341–*GUS* and B341M3–*GUS* plants. Incubation was in the presence of different concentrations of auxin. Miniexplants represent a tissue culture system particularly suitable for the analysis of auxin response of *rolB* (Maurel, 1991; Bellincampi et al., 1996), because expression of the gene in the leaf blade is detectable only in the main veins (Capone et al., 1991; see below) and is therefore initially very low in miniexplants. Figure 4A shows the dose–response curve for the different *GUS* constructs after 48 hr of exposure of the miniexplants to auxin. This time period allows optimal measurement of auxin-induced GUS activity in miniexplants (Bellincampi et al., 1996). Whereas the low initial activity of the B341–*GUS* contructs showed the expected strong stimulation by auxin, B341M3–*GUS* did not respond to any of the concentrations of the hormone used.

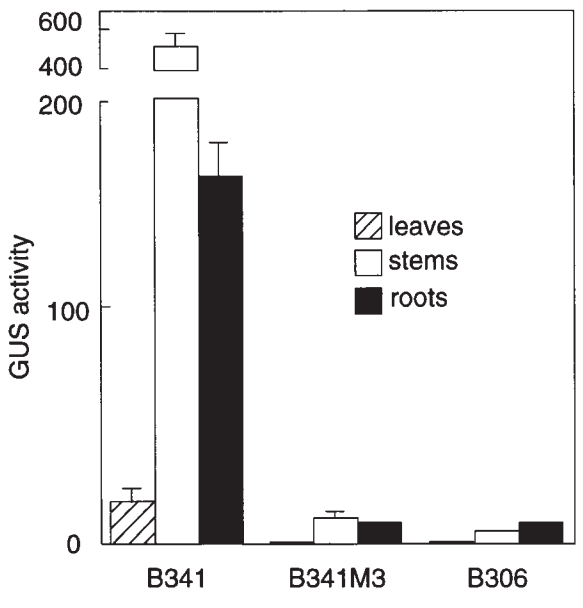

Figure 3. Level of Activity of *rolB–GUS* Fusions in Different Organs of Tobacco Plants.

The bars in the histogram represent averages (±SE) of four independent measurements, using extracts from different in vitro–grown T_2 plants. GUS activity is reported as picomoles of 4-methylumbelliferone per milligram of protein per minute.

NtBBF1 and *rolB* Have Similar Expression Patterns

The data presented above indicate that the target sequence of NtBBF1 within domain B plays a key role in the regulation of *rolB* expression. We then analyzed the pattern of expression of *NtBBF1* to assess its compatibility as that of a regulatory gene of *rolB.*

Figure 5A shows the hybridization of a probe spanning 442 bp at the 3′ end of *NtBBF1* with total RNA from different tobacco organs. To avoid hybridization with mRNAs of other members of the tobacco Dof gene family, the probe did not include the conserved Dof domain, as shown in the schematic representation of *NtBBF1* in Figure 5C. As shown in Figure 5A, expression of *NtBBF1* was detectable in all organs tested. These included leaves, stems, and roots, with staining being most intense in roots.

To analyze in more detail the pattern of *NtBBF1* expression, tobacco plants were transformed with transcriptional fusions of the *GUS* gene with two segments of the 5′ noncoding region of *NtBBF1*, spanning 2231 bp (BBF2231–*GUS*) and 814 bp (BBF814–*GUS*), respectively. The results obtained with the two constructs are identical. Thus, only those results relative to the shorter construct, BBF814–*GUS*, are presented. With respect to apical meristems, Figure 2C shows that the expression of *NtBBF1* in the primary root apex is limited to the meristematic region, closely matching the pattern of *rolB* expression shown in Figure 2A. The expression pattern of *NtBBF1* is also very similar to that of *rolB* in the primordia of secondary roots, as can be seen by comparing Figures 2F and 2D. A comparison of Figures 2I and 2G shows that the same also holds true for the shoot meristem in which both genes are very actively expressed.

In Figure 6, the GUS staining pattern due to the *rolB* (B341–*GUS*) and *NtBBF1* (BBF814–*GUS*) constructs in stems and leaves is shown. In stems, the expression patterns of *rolB*, as shown in Figure 6A, and of *NtBBF1*, as shown in Figure 6B, appeared to be very similar and are localized in the phloem and in the phloem and xylem parenchyma. Figures 6C and 6D show the very similar expression patterns of *rolB* and *NtBBF1*, respectively, in cotyledons of plantlets 1 week after germination. In both cases, GUS activity was limited to the main vasculature. Expression of the two genes also was very similar and limited to the vasculature in the small leaves of plantlets 3 weeks after germination (data not shown). In more mature leaves (4 to 5 cm long from in vitro–grown plants), the activity of the B341–*GUS* construct was still limited to the veins (Figure 6E), whereas the activity of BBF814–*GUS*, although higher in the vascula-

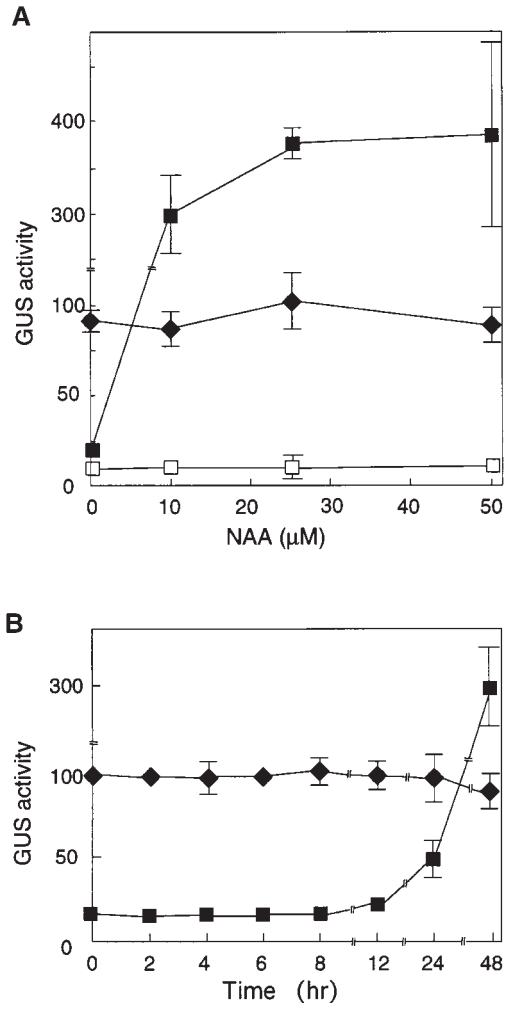

Figure 4. Mutation of the NtBBF1 Binding Sequence Suppresses Auxin Activation of the *rolB* Promoter.

Auxin dose–response **(A)** and kinetics of auxin effects **(B)** on GUS activity of different *GUS* contructs.

(A) Leaf miniexplants were incubated for 48 hr in liquid medium in the presence of the 1-naphthaleneacetic acid (NAA) concentrations indicated.

(B) Leaf miniexplants were incubated for 48 hr in liquid medium for different lengths of time in the presence of 10 μM NAA.

For each NAA concentration and time point, the average of the GUS activity (±SE) of extracts from two pools of several leaves from different in vitro–grown T_2 plants from two different T_1 parents is reported in picomoles of 4-methylumbelliferone per milligram of protein per minute. Filled squares, B341–*GUS*; open squares, B341M3–*GUS*; diamonds, BBF814–*GUS.* The *NtBBF1* gene is not auxin inducible.

ture, also was histochemically detectable in the mesophyll (Figure 6F).

NtBBF1 Is Not Inducible by Auxin

We assayed the auxin inducibility of the promoter of *NtBBF1* by using the leaf miniexplant system described above. Figure 4A shows that in contrast to that in B341–*GUS*, GUS activity of leaf extracts from in vitro–grown BBF814–*GUS* plants was moderate but also significant in the absence of exogenous auxin (in agreement with the histochemical staining of the mesophyll of these leaves shown in Figure 6F). However, as shown in Figure 4A, this activity did not increase when miniexplants were exposed to different concentrations of the hormone for 48 hr. GUS activity of miniexplants from leaves of plants transformed with the longer construct BBF2231–*GUS* also were found to be auxin insensitive (data not shown).

To test the possibility of a short-term transient induction of *NtBBF1* by auxin, we assayed GUS activity of the miniexplants after different times of incubation with the hormone. The data reported in Figure 4B confirm the slow kinetics of the strong activation of the *rolB* promoter by auxin (Maurel et al., 1990, 1994; Capone et al., 1991) and show the lack of response of the *NtBBF1* promoter between 2 and 48 hr after hormone treatment. Accordingly, no auxin-responsive elements (AuxREs) could be identified by sequence analysis of the >2 kb of *NtBBF1* promoter used in this work.

To verify these reporter gene data, we analyzed the steady state level of *NtBBF1* mRNA by RNA gel blot analysis of leaf miniexplants 48 hr after incubation in the absence and presence of exogenous auxin. The results are shown in Figure 5B. The intensity of the *NtBBF1* mRNA autoradiographic bands in the absence and presence of the hormone are 0.57 and 0.40, respectively, of the actin signal in the same well, confirming the lack of auxin inducibility of *NtBBF1.*

Assaying Ectopic Expression of *rolB* by Using Particle Gun Delivery

The above-described discrepancy of the *NtBBF1* and *rolB* expression patterns in mature leaves may imply that NtBBF1 is not the only *trans*-acting protein involved in the control of *rolB* expression. Despite the presence of NtBBF1, the activity of the *rolB* promoter in the mesophyll of mature leaves might be maintained below the threshold of sensitivity of the histochemical assay by a negative regulatory factor present in these cells. Increasing the number of copies of the *rolB* promoter might alter the balance of positive and negative factors and result in ectopic expression of the oncogene. We tested this possibility by assaying the transient expression of different *GUS* fusions by using particle gun bombardment.

Constructs B341–*GUS*, B341M3–*GUS*, B306–*GUS*, and a *GUS* fusion with the cauliflower mosaic virus 35S promoter

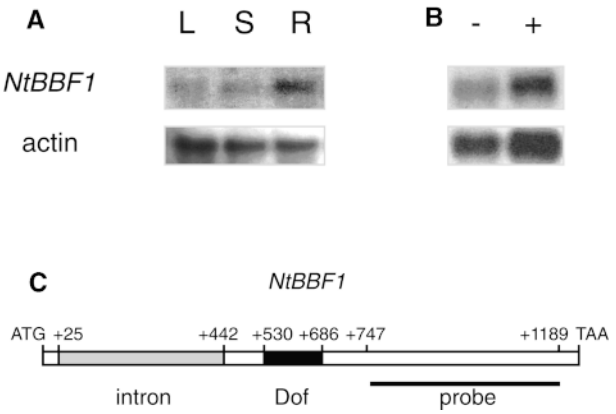

Figure 5. *NtBBF1* Is Expressed in Different Organs of Tobacco Plants and Is Not Inducible by Auxin.

(A) RNA gel blot analysis of total RNA from leaves (L), stems (S), and roots (R) of greenhouse-grown SR1 tobacco plants.
(B) RNA gel blot analysis of poly(A)-containing RNA from SR1 leaf miniexplants incubated for 48 hr in the absence (−) and presence (+) of 10 μM NAA. The probes are derived from the *NtBBF1* and actin genes.
(C) The bar is a schematic representation of the *NtBBF1* gene (EMBL accession number AJ009594). The numbers indicate nucleotide positions from the translation start codon. The positions and lengths of the single intron of the Dof domain and of the probe used in the RNA gel blots are indicated. TAA is the first in-frame stop codon.

(35S–*GUS*) were delivered to leaves (4 to 5 cm long) of in vitro–grown untransformed tobacco plants. The histogram in Figure 7 shows the results of the particle gun transient assays. Delivery of the 35S–*GUS* construct resulted, after histochemical staining, in a very large number of GUS-positive spots on bombarded leaves, typically ~500, as expected from the strength and constitutive activity of the viral promoter in all plant tissues. Numerous spots also were reproducibly observed in the mesophyll of leaves bombarded with construct B341–*GUS* (weaker than 35S–*GUS*; Capone et al., 1991, 1994), indicating ectopic activity of the *rolB* promoter. As compared with those of B341–*GUS*, the number of spots is greatly reduced when the target sequence of NtBBF1 within domain B is modified (B341M3–*GUS*) or domain B is completely deleted (B306–*GUS*).

The particle gun delivery experiments with the 35S–*GUS*, B341–*GUS*, and B341M3–*GUS* constructs also were performed with small leaves of plantlets 2 to 3 weeks after germination. In these leaves, *NtBBF1* and *rolB* are both expressed only in the vasculature. The inset in Figure 7 shows that in young leaves, the number of spots due to delivery of the B341–*GUS* construct is substantially lower than in mature leaves and drops almost to the level observed with construct B341M3–*GUS.*

DISCUSSION

A DNA Regulatory Motif Necessary for Tissue Specificity and Involved in Auxin Response

In this study, we show that the target sequence of the DNA binding protein NtBBF1 is a *cis*-regulatory element that mediates the tissue specificity of expression of the oncogene *rolB* in plants (Schmülling et al., 1989; Maurel et al., 1990; Altamura et al., 1991; Capone et al., 1991) and also is involved in its inducibility by auxin (Maurel et al., 1990, 1994; Capone et al., 1991, 1994).

The modification of the ACTTTA binding sequence of NtBBF1 within the regulatory domain B in the promoter of *rolB* virtually suppresses gene expression in apical meristems. The low fluorescence values measured in whole organs of B341M3–*GUS* plants point to a role for the ACTTTA motif in controlling the level of expression of the oncogene in the vascular system as well. In the stem, leaves, and to a lesser extent in roots, the fluorescence due to *rolB–GUS* is, in fact, quantitatively accounted for basically by the vascular system (Capone et al., 1991).

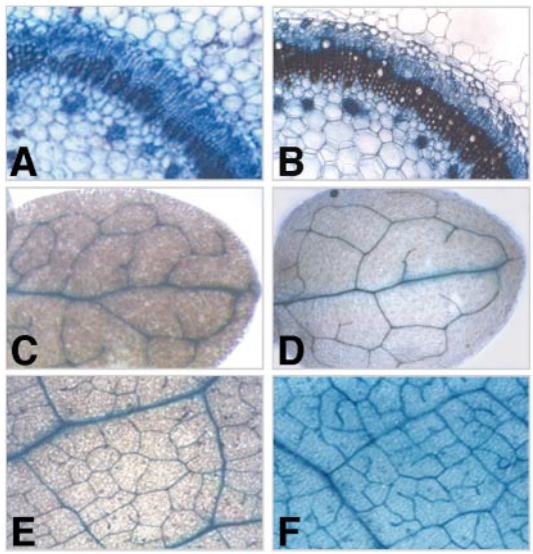

Figure 6. *rolB* (B341–*GUS*) and *NtBBF1* (BBF814–*GUS*) Have Similar Expression Patterns, Except in Mature Leaves.

(A), **(C)**, and **(E)** Activity of the B341–*GUS* construct in the stem (transverse section), cotyledon, and leaf blade, respectively, of tobacco plants.
(B), **(D)**, and **(F)** Activity of the BBF814–*GUS* construct in the stem (transverse section), cotyledon, and leaf blade, respectively, of tobacco plants.
(C) and **(D)** show T_2 plantlets 1 week after germination; **(A)**, **(B)**, **(E)**, and **(F)** show in vitro–grown T_2 plants.

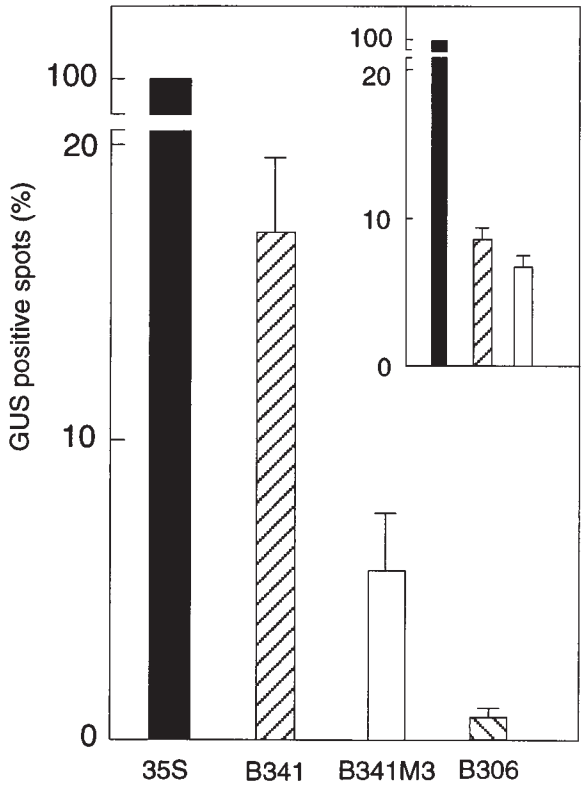

Figure 7. Transient Ectopic Expression of *rolB* in Mesophyll Cells Depends on the NtBBF1 Binding Sequence and on *NtBBF1* Expression.

The number of GUS-positive spots observed in the mesophyll of mature leaves from in vitro–grown SR1 plants was determined after particle gun delivery of constructs 35S–*GUS*, B341–*GUS*, B341M3–*GUS*, and B306–*GUS*. Each bar represents the average (±SE) of measurements of 10 leaves (4 to 5 cm long from different plants) that were each bombarded twice with a given construct. The number of spots is normalized to that observed with the 35S–*GUS* construct (typically ~500 spots) in the same round of bombardments with the different constructs. The inset shows delivery of 35S–*GUS*, B341–*GUS*, and B341M3–*GUS* to small leaves of SR1 plantlets 2 to 3 weeks after germination.

The NtBBF1 binding motif seems to be one of the regulatory sequences involved in the responsiveness of the *rolB* promoter to auxin. The overall reduction of activity of the mutated B341M3 promoter does not seem to justify its lack of inducibility by the hormone. In a previous analysis of the *rolB* promoter, we reported that constructs carrying internal deletions between position −341 and the translation start codon show reduced GUS activity but are still inducible by auxin, if domain B is present (Capone et al., 1994). The same analysis also pointed to several other regulatory domains in the B341 promoter (domains C, D, and E) and showed that domain B is not sufficient per se for conferring auxin responsiveness to a minimal promoter (Capone et al., 1994). Thus, the ACTTTA sequence in domain B is one of the regulatory elements necessary for auxin responsiveness of *rolB*; however, other sequences also are critical for the regulation of this complex promoter.

Our data show that the binding sequence of a member of the Dof family of plant zinc finger proteins plays a role in regulating gene expression in the plant. Members of this new family of plant proteins characterized by a highly conserved domain comprising a single zinc finger (the Dof domain) have been identified independently by different authors in phylogenetically very distant plants (Yanagisawa and Izui, 1993; Kisu et al., 1995; Zhang et al., 1995; De Paolis et al., 1996; Vicente-Carbajosa et al., 1997; Mena et al., 1998). In contrast, Dof proteins are not present in yeast or in animals (Yanagisawa and Sheen, 1998). The wide (possibly universal) distribution of these proteins only in plants suggests that they are involved in regulatory circuits specific to the plant kingdom. The role of the ACTTTA motif in the regulation of *rolB* substantiates this hypothesis. Recently, other members of the Dof protein family have been suggested to be involved in light-regulated gene expression (Yanagisawa and Sheen, 1998) and in the regulation of storage protein genes in cereals (Mena et al., 1998).

The fact that the ACTTTA motif is necessary for tissue-specific expression and also seems to be involved in the response to auxin establishes an intriguing connection between these two aspects of the regulation of *rolB*. It must be recalled that during zygotic embryogenesis, *rolB* is activated at the end of the globular stage (Chichiriccò et al., 1992), when embryo cells acquire the capability to respond to auxin (Lo Schiavo et al., 1991). This activation and the fact that the spatial pattern of *rolB* expression in transgenic plants is modified by treatment with exogenous auxin (Maurel et al., 1990) suggest a link between hormonal and developmental control of *rolB* expression (Maurel et al., 1990; Binns and Costantino, 1998). The data presented here strengthen this hypothesis. Developmental control and cell-specific expression of the oncogene might thus be due to differences in hormone concentration and/or sensitivity in different cell types and at different stages of development.

The NtBBF1 Protein Is Very Likely To Be Involved in *rolB* Regulation

The data presented in this work also point to NtBBF1 as the *trans*-acting factor necessary for the control of *rolB* expression mediated by the ACTTTA *cis* element. The almost perfect overlap of the expression patterns of the *NtBBF1* and *rolB* genes is in fact strongly suggestive of a role of the former in regulating the latter. In addition, the protein NtBBF1 was identified initially because of its binding to the ACTTTA sequence, and *NtBBF1* was the only positive cDNA clone identified in a tobacco expression library of >150,000 plaques screened with a tetramer of domain B of the *rolB*

promoter (De Paolis et al., 1996). This strongly suggested that NtBBF1 corresponds to the tobacco nuclear protein shown to bind to domain B (De Paolis et al., 1996).

However, the high degree of conservation of the zinc finger domain among the Dof proteins suggests that they may all recognize related DNA sequences. Indeed, maize Dof1 recognizes a CCTTTT motif (Yanagisawa and Izui, 1993; Yanagisawa and Sheen, 1998), and the maize PBF protein and its barley BPBF counterpart bind a CTTTA sequence (Vicente-Carbajosa et al., 1997; Mena et al., 1998). The target sequence of the Arabidopsis Dof protein OBP1 includes a CTTT core (Chen et al., 1996). In addition, an Arabidopsis protein isolated in our laboratory, AtBBFa (De Paolis et al., 1996), is capable of binding, at least in vitro, the ACTTTA motif (M. Papi and P. Costantino, unpublished results).

Thus, the specificity of the regulatory roles of the individual Dof proteins within a plant, rather than a promoter-specific DNA target site, may rest with other determinants, such as the tissue-specific availability of these proteins and/or their interaction with other regulatory factors. The *Dof1* and *Dof2* genes are indeed expressed differentially in maize (Yanagisawa and Sheen, 1998), and their gene products interact with each other (Yanagisawa, 1997). The maize Dof protein PBF is endosperm-specific and interacts with the basic leucine zipper (bZip) protein Opaque2 (Vicente-Carbajosa et al., 1997). In Arabidopsis, the Dof protein OBP1 interacts with OBF, which is a bZIP protein that recognizes the *ocs* element in the promoter of the glutathione *S*-transferase-6 gene (Zhang et al., 1995; Chen et al., 1996). In addition, the Arabidopsis genes *AtBBFa* and *AtBBFb* and the other tobacco *Dof* genes *NtBBF2a* and *NtBBF3* isolated in our laboratory all show distinctive expression patterns, different from that of *NtBBF1* reported here (S. Sabatini, P. Vittorioso, and P. Costantino, unpublished results). In summary, tissue specificity and/or interactions with other regulatory factors seem to be general features of the Dof zinc finger proteins. From this perspective, the expression pattern of the *NtBBF1* gene, almost perfectly overlapping that of *rolB*, strongly points to NtBBF1 as being a regulator of *rolB*.

The results of the particle gun delivery experiments presented here also support this suggestion and provide indirect evidence that an additional regulatory factor (possibly interacting with NtBBF1) may mediate the tissue specificity of expression of *rolB.* The only gross discrepancy between the patterns of expression of *NtBBF1* and *rolB* is, in fact, observed in the mesophyll of mature leaves: here, the former is expressed and the latter is not. This discrepancy of expression prompted the particle gun delivery experiments, whereby ectopic expression of *rolB* in the mesophyll of mature leaves could be achieved, possibly by titrating a putative negative factor as a consequence of the great increase in the number of copies of the oncogene. This ectopic expression depends on binding of the Dof protein present in the mesophyll, as shown by the substantial reduction in the number of GUS-positive spots observed when leaves are bombarded with the *GUS* construct mutated in the NtBBF1 binding sequence. Accordingly, transient *GUS* expression driven by the B341 promoter in mesophyll tissue drops almost to that of mutated B341M3 when young leaves (in which *NtBBF1* is expressed only in the vasculature) are bombarded.

NtBBF1 and *rolB* Auxin Induction

In transformed plant cells and tissues, *rolB* is specifically induced by active auxins (Maurel et al., 1990) with rather slow kinetics. The earliest detectable increase in the activity of *GUS* fusions (Maurel et al., 1990; Capone et al., 1991) or in the level of *rolB* mRNA (Maurel et al., 1994) occurs after 6 to 8 hr of hormone treatment. Thus, the oncogene does not belong to the class of early (or primary) genes rapidly induced by auxin. These genes play a role in the transcriptional regulation of late-responsive (secondary) genes (Abel and Theologis, 1996). Rather, *rolB* belongs to this secondary class of genes that determines a specific biological response as a long-term consequence of the auxin stimulus.

Early auxin-induced genes have attracted considerable attention: AuxREs have been identified (reviewed in Abel et al., 1996), and a *trans*-acting auxin response factor capable of binding to AuxREs has been isolated recently (Ulmasov et al., 1997). In contrast, little is known about late-responsive genes and their *cis*- and *trans*-regulatory elements and about the the mechanisms involved in mediating long-term auxin effects on gene expression. The identification of one of the *cis* elements involved and of the Dof regulatory protein interacting with it provides an important tool for clarifying the auxin response of *rolB* and for shedding light on the pathway leading to the specific biological response from the initial hormonal stimulus. Interestingly, another Dof factor, AOBP, binds to the promoter of the pumpkin ascorbate oxidase gene (Kisu et al., 1995), another late auxin-responsive gene (Esaka et al., 1992).

In the case of *rolB*, the long-term effect of auxin is on organogenesis (normally involving roots but also, at least in vitro, shoots and flowers) due to stimulation of meristem formation (Altamura et al., 1994), which is in turn due to auxin-induced expression of the oncogene (Bellincampi et al., 1996). Meristem formation is triggered by a 10^3- to 10^4-fold increase in sensitivity to auxin of plant cells (Spanò et al., 1988; Maurel et al., 1991), possibly due to the tyrosine phosphatase activity of the RolB protein (Filippini et al., 1996), which may perturb the auxin signal transduction pathway. Oligogalacturonides, a class of oligosaccharins with anti-auxin effects (Darvill et al., 1992; Bellincampi et al., 1993), inhibit auxin induction of *rolB* (Bellincampi et al., 1996).

This model is diagrammed in Figure 8. In this scheme, the isolation of NtBBF1 will help to address crucial questions, such as how *rolB* is induced by auxin, the way in which oligogalacturonides interfere with this process, and which plant genes are triggered by NtBBF1.

The level of expression of the *NtBBF1* gene is proven here

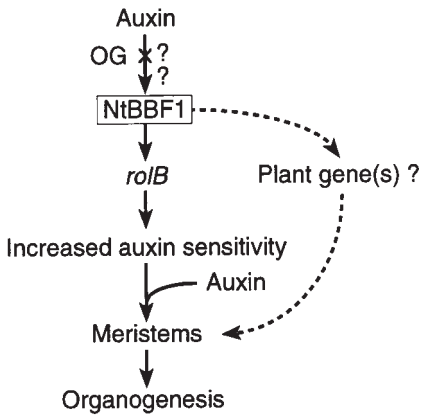

Figure 8. Schematic Model of Auxin-Induced *rolB*-Mediated Organogenesis.

Auxin induces expression of *rolB*; NtBBF1 is necessary for induction; oligogalacturonides (OG) inhibit induction. The RolB protein causes a 10^3- to 10^4-fold increase in the sensitivity to auxin of plant cells. This increased auxin sensitivity is possibly the trigger for de novo formation of meristems. The type of meristem (and organ) formed depends on local hormonal balance and/or cell competence. Question marks indicate issues that can now be directly addressed on the basis of the results presented in this work.

not to be sensitive to auxin, ruling out the possibility that the slow response of *rolB* is mediated by a direct transcriptional control of the hormone on *NtBBF1.* This opens the intriguing possibility that auxin may directly or indirectly affect the activity and/or the stability of the NtBBF1 protein. Alternatively, the hormone may control a putative negative regulatory factor, as shown by the results of the particle gun experiments.

It will be interesting to identify the factor(s) interacting with NtBBF1 in the control of tissue-specific and hormonal responses of *rolB* as well as the plant gene(s) that is the normal regulatory target of NtBBF1. In fact, although *rolB* is an oncogene of exogenous (bacterial) origin, its promoter is very likely a mosaic of plant *cis* regulatory elements, which interact with plant-encoded proteins (such as NtBBF1). Therefore, we suggest that the Dof protein NtBBF1 may be a regulatory factor involved in tissue-specific expression and possibly the auxin response of endogenous plant genes.

METHODS

Reporter Gene Fusions

Transcriptional fusions of the *rolB* promoter deletions B341 and B306 with the β-glucuronidase (*GUS*) reporter gene, both in plasmids pUC18 (used in this work for particle gun experiments) and pBI (used for transformation), were previously described (Capone et al., 1991). The mutagenized version B341M3–*GUS* was derived from the intermediate construct of B341 in pUC18 (Capone et al., 1991) by substituting the 50-bp HindIII fragment with the synthetic oligonucleotide 5′-AGCTTGTCGACGGTCCCGCTTTCGCGAAATCCAATAGCGGGCCATGATTA-3′, in which the ACTTTA sequence within domain B is changed to TGATTA (see underlining). The mutagenized promoter was then cloned as a SalI-BamHI fragment in pBI101.2. The transcriptional fusion BBF2231–*GUS* was derived from the λ*NtBBF1g* clone (a 4-kb EcoRI genomic clone in pBluescript II KS+ and KS− [Stratagene, La Jolla, CA]; De Paolis et al., 1996) by digesting with BsmBI, filling in with the Klenow fragment of DNA polymerase I, digesting with SmaI, and ligating to obtain a clone (BBF2231–pBluescript) spanning nucleotides −2261 to −30 from the ATG start codon of *NtBBF1.* This promoter segment was then cloned as a SalI-BamHI fragment in pBI101 to obtain BBF2231–*GUS.* Construct BBF814–*GUS* was derived from clone BBF2231–pBluescript by digestion with HindIII and ligation; the promoter was then cloned as a HindIII-BamHI fragment in pBI101.

Plant Material

Petit Havana SR1 tobacco (*Nicotiana tabacum*) plants (Maliga et al., 1973) were grown in vitro on Murashige and Skoog basal medium (Murashige and Skoog, 1962; Sigma) with a 16-hr illumination period at 25°C and propagated monthly by cutting. T_1 transgenic plants were obtained by leaf disc infections and selection on 300 μg/mL kanamycin. Regenerants were grown in vitro (in the text, "in vitro–grown" indicates plants propagated 2 or 3 times 4 weeks from the last transfer onto fresh medium), transferred to an illuminated (16-hr illumination period), temperature-controlled (20 to 24°C) greenhouse, and allowed to self-fertilize. To obtain T_2 plants, T_1 seeds were germinated on Murashige and Skoog medium and selected on 300 μg/mL kanamycin. T_2 plants were grown as described for T_1 plants.

GUS Assays

Histochemical staining and microscopic analysis were conducted as described previously (Capone et al., 1991), except that before overnight staining, the samples were vacuum infiltrated for 5 min, and 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ were added to the staining solution. Unfixed whole plantlets, organs, or hand-cut sections were examined and photographed under a Leitz Laborlux D light microscope (Leitz, Wetzlar, Germany). In Figures 2 and 6, typical representatives of staining patterns relative to each construct in different T_2 plants from at least five independent T_1 transformants are shown.

Fluorometric assays with extracts from organs and leaf miniexplants were performed as previously described (Capone et al., 1991; Bellincampi et al., 1996). All fluorescence values were normalized for protein content.

Leaf Miniexplants

Leaves 4 to 5 cm long from in vitro–grown T_2 plants were used to prepare leaf miniexplants (~2 × 2 mm) that were devoid of primary and secondary veins (Maurel, 1991; Bellincampi et al., 1996). Batches of 15 miniexplants were incubated in 3 mL of Murashige and Skoog liquid medium supplemented with 2% sucrose in the presence of various concentrations of 1-naphthaleneacetic acid (Sigma) for up to 48 hr under a 16-hr illumination period at 25°C.

RNA Gel Blot Analysis

Total RNA was prepared from leaves, stems, and roots of greenhouse-grown SR1 plants (~25 cm high) and from SR1 leaf miniexplants. Samples were frozen in liquid nitrogen, and RNA was isolated using the RNeasy plant minikit (Qiagen, Chatsworth, CA). Samples of total RNA (10 μg) or poly(A)-containing RNA (2 μg purified on Dynabeads Oligo(dt) [DYNAL, Oslo, Norway]) were separated by electrophoresis on formaldehyde–agarose gels and transferred to a hybridization membrane (Hybond C-extra; Amersham). The *NtBBF1* probe is a 442-bp DNA fragment (nucleotides 747 to 1189) from the 3′ portion of the *NtBBF1* gene that does not encompass the Dof domain. To normalize for differences in the amounts of RNA loaded, filters were also hybridized with a 1054-bp fragment of the tobacco actin cDNA (kindly provided by R. van Aarssen, Gent, Belgium). The relative intensities of the autoradiographic signals were assessed by scanning the films on a Bio-Rad GS-670 imaging densitometer.

Particle Gun Delivery Assays

Leaves (4 to 5 cm long) from in vitro–grown SR1 plants or young leaves from 2- to 3-week-old SR1 plants were placed on 9-cm agar/Murashige and Skoog Petri dishes and bombarded twice with 1.25 μg of DNA of the different promoter–*GUS* fusions in plasmid pUC18, using a BioRad PDS/1000 He apparatus (chamber vacuum of 25 inches Hg; helium pressure of 1300 psi; target distance of 9 cm). Leaves were analyzed for histochemical GUS staining 24 hr after bombardment.

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